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Enteroviral Polymerase Chain Reaction in the Investigation of Aseptic Meningitis

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We used a nested polymerase chain reaction (nPCR) to seek evidence for enteroviruses in clinical samples from patients with symptoms of aseptic meningitis. When compared with conventional virus isolation methods on a total of 366 samples collected during 1994–1995, an increase in positivity from 6% to 27% was shown. The results indicate that nPCR would be a valuable aid to the laboratory diagnosis of enteroviral infections as it can detect those enteroviruses that cannot be identified by current isolation methods.

KEY WORDS: enteroviruses, meningitis, CNS, nested PCR

INTRODUCTION

The human enteroviruses are a major worldwide cause of viral meningitis. Almost all the coxsackie A and B viruses and most echoviruses, in addition to polioviruses, have been associated with aseptic meningitis [Melnick, 1990]. Evidence for this association has accumulated over the years based on the use of virus isolation in cell culture and subsequent neutralisation using pooled antisera [reviewed by Minor and Bell, 1990]. This practice, however, fails to identify the many enterovirus serotypes that do not grow well in cell culture and are rarely, if ever, isolated. Recently, PCR has been described as a valuable approach to detect enteroviral RNA in muscle biopsies, cerebrospinal fluid (CSF), stool, throat swabs, and serum samples and has emerged as a potential diagnostic test [Glimaker, 1992; Glimaker et al., 1992; Kammerer et al., 1994; Leparc et al., 1994; Nicholson et al., 1994; Thoren and Widell, 1994]. As almost all enteroviral serotypes have a conserved 5′ nontranslated region (NTR), using the polymerase chain reaction (PCR) with primers from this region offers a means of identifying these viruses using one assay [Gow et al., 1991; Zoll et al., 1992]. We used nested PCR (nPCR) to examine specimens submitted to the Regional Virus Laboratory from January 1994 to December 1995 inclusive from patients with symptoms of aseptic meningitis and compared the results with conventional virus isolation methods.

MATERIALS AND METHODS

Patients

This study was conducted throughout 1994 and 1995 on specimens from patients with symptoms of aseptic meningitis based on clinical information on the laboratory request card and in which enough specimen remained after routine viral isolation. A total of 366 specimens (140 CSF, 181 throat swabs, 45 stools) from 340 patients were available for study. The study population consisted of 160 males (average age 22.9 years, age range 4 months to 56 years) and 180 females (average age 24.2, age range 4 months to 50 years).

Virus Isolation and Control Preparation

Virus isolation was undertaken on all specimens using monolayered cultures of rhesus monkey kidney, MRC5, and Hep-2 cells. Tube cultures were inoculated with 0.2 ml specimen (10% stool suspension), incubated at 37°C, and observed for cytopathic effect for up to 14 days. Virus isolates were then typed by neutralisation test [Grist et al., 1979]. The positive control specimen was tissue culture fluid from MRC5 cells infected with coxsackie A9 virus. To determine the limit of detection of the PCR with virus culture, four replicate log dilutions of coxsackie A9 virus were titrated and tested to establish the 50% tissue culture infectivity dose ($TCID_{50}$) in cell culture and the lowest dilution with positive results by PCR.

PCR

RNA was extracted from 200 μl specimen using TRIzol reagent as described by the manufacturer. PCR was performed as previously described with nested oligonucleotide primers designed in the highly conserved 5′ NTR of the enterovirus genome in order to obtain a broad reactivity with this group of viruses [Galbraith et al., 1995; Gow et al., 1991]. The amplification of human

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TABLE I. Comparison of nPCR and Viral Culture on Clinical Specimens

Specimen	1994 nPCR+	1994 Culture+	1994 Number tested	1995 nPCR+	1995 Culture+	1995 Number tested
CSF	20	2	58	15	4	82
Throat swab	19	4	49	28	7	132
Stool	6	2	11	11	3	34
Total	45	8	118	54	14	248

Abelson tyrosine kinase gene provided a check on the quality of RNA extracted and only those samples that gave a positive amplification product were included [Hermans et al., 1988]. PCR products were visualised on a 2.5% agarose gel stained with ethidium bromide. At each stage of the PCR, positive and negative controls (coxsackie A9 virus and MRC5 cells, respectively) were included and must have shown the expected results for the test to be valid. All samples were assayed blind to eliminate bias.

RESULTS

The sensitivity of detection was assessed using serial dilutions of the positive control material and proved to be between a 1×10^3 and 1×10^7 dilution of coxsackie A9 virus. The nested primers were 1,000-fold more sensitive than the first-round primers and detected control coxsackie A9 enteroviral RNA at a dilution of 1×10^7 which was equivalent to 0.0001 $TCID_{50}$ of control virus.

Results of nPCR on clinical samples are summarised in Table 1 together with the virus isolation results. Of the total 366 samples tested, 27% were positive by nested enteroviral PCR whereas only 6% were positive by virus isolation. In general terms, the results in 1994 and 1995 are comparable. Enterovirus RNA was detected most commonly in stool samples (38% positive), but less frequently from throat swabs and CSF (26% and 25% positive, respectively). All samples from which virus was isolated were PCR positive. The clinical isolates were identified by neutralisation as echovirus types 4, 5, 6 (three isolates), 7 (two isolates), 9 (six isolates), 11 (two isolates), coxsackie A9 (three isolates), coxsackie B2, and herpes simplex virus type 1 isolated from a throat swab. Two of the cultured enteroviruses could not be typed by neutralisation.

The seasonal distribution of the nPCR positive samples and the number of virus isolations from patients during the 2 years of the study are shown in Figure 1. Although the number of enteroviruses detected by nPCR showed a peak in the third quarter of the year when 38% of the samples tested were positive, the remaining positive specimens were identified throughout the year. During the months July to September, 52% of the isolates were identified by culture.

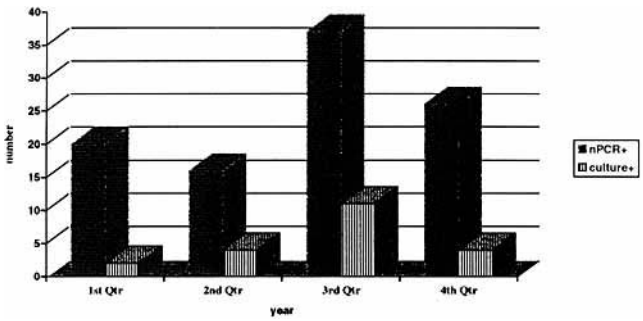

Fig. 1. Seasonal distribution of positive nPCR and culture results for 1994 and 1995 combined.

DISCUSSION

This study has shown that improvements in the diagnosis of aseptic meningitis can be made both in accuracy and speed. By using the traditional virus isolation approach, 6% of the samples from patients with suspected aseptic meningitis were positive whereas 27% were positive by nPCR. The potential has therefore been shown for a more precise diagnosis. Currently, in up to 80% of cases of suspected aseptic meningitis the causative agent remains undetermined. The importance of an accurate diagnosis of the cause of meningitis cannot be underestimated and it is valuable to be able to distinguish aseptic meningitis due to enteroviral infection from bacterial meningitis which can present with a similar clinical picture [Glimaker, 1992].

PCR was compared with the currently available virus isolation techniques used to detect enteroviruses in patients with symptoms of suspected aseptic meningitis who were referred to the Regional Virus Laboratory from the West of Scotland. It clearly demonstrated an increased sensitivity over virus isolation. These results are consistent with earlier findings [Kammerer et al., 1994; Leparc et al., 1994; Nicholson et al., 1994; Thoren and Widell, 1994]. All specimens from which enterovirus was isolated were also PCR positive. In one throat swab, herpes simplex type 1 was isolated and enteroviral RNA detected. This is not unusual and represents a dual infection. An enterovirus in the presence of a herpes simplex virus would be difficult to identify by neutralisation.

The nPCR turn around time was in all cases superior to isolation techniques as typically a culture can take up to 14 days for a cytopathic effect to develop whereas nPCR results are available in 48 hours, and costs were comparable. We suggest that the nPCR technique could be used as a screening test to identify enterovirus in appropriate specimens from patients with suspected aseptic meningitis. For confirmation, the specimen could subsequently be inoculated to culture the virus and iden-

tify the specific serotype involved in the meningitis. This would reduce the number required to be cultured for enteroviruses by 73% (from 366 to 99). However, infection due to other viruses such as herpes simplex virus type 1 would still need to be excluded.

An important advantage of using PCR is that those enteroviruses which are unable to be grown in culture may be amplified and then typed by direct sequencing of PCR products and phylogenetic analysis [Arola et al., 1996]. Another advantage of PCR is the detection of enteroviral sequences in situations where the virus in the clinical sample may not be viable, for example, after prolonged maintenance at room temperature during transport.

Although detection of enterovirus in CSF by isolation is formally required for the diagnosis of an enteroviral aseptic meningitis, additional information can be obtained using specimens from other sites such as throat swabs and stool if CSF is not available. Comparative studies of information obtained in cases of aseptic meningitis using PCR and culture on samples obtained from different anatomical sites have so far only been performed in relatively small patient groups with meningitis [Kammerer et al., 1994; Nicholson et al., 1994; Thoren and Widell, 1994]. Currently we are extending these studies.

It was interesting to note that the nPCR showed a year-round distribution of enteroviruses associated with aseptic meningitis rather than the predominance in the summer and autumn months typically observed with cell culture isolations [Melnick, 1990; Minor and Bell, 1990]. This suggests that enteroviral infections may be less seasonally distributed than previously thought.

In conclusion, if nPCR was applied to the diagnosis of enteroviral meningitis, a far higher number of enterovirus infections would be reported, the clinical diagnosis would be more accurate, and resources more appropriately managed. Thus, our laboratory introduced nPCR for enteroviruses in cases of aseptic meningitis as a reliable routine diagnostic test in January 1996.

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